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<b>(21) International Application Number:</b> PCT/DK97/00230 <b>(22) International Filing Date:</b> 20 May 1997 (20.05.97)  <b>(30) Priority Data:</b> 0585/96                      20 May 1996 (20.05.96)                      DK  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> NIELSEN, Per, Munk [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).  <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD OF OBTAINING PROTEIN HYDROLYSATES  <b>(57) Abstract</b>  The present invention relates to a method of obtaining protein hydrolysates useful as flavoring agents. More specifically, the invention provides a method of obtaining from a proteinaceous substrate a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, which method comprises the steps of subjecting the substrate to a deamidation process, and subjecting the substrate to the action of a specific acting proteolytic enzyme.		

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## A METHOD OF OBTAINING PROTEIN HYDROLYSATES

### TECHNICAL FIELD

5       The present invention relates to a method of obtaining protein hydrolysates useful as flavoring agents. More specifically, the invention provides a method of obtaining from a proteinaceous substrate a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, which method comprises the steps of  
10       subjecting the substrate to a deamidation process, and subjecting the substrate to the action of a specific acting proteolytic enzyme.

### BACKGROUND ART

      Various food products, e.g. soups, sauces and seasonings, comprises  
15       flavoring agents obtained by hydrolysis of proteinaceous materials. Conventionally this hydrolysis is brought about using strong hydrochloric acid, followed by neutralization with sodium hydroxide. However, such chemical hydrolysis leads to severe degradation of the amino acids obtained during the hydrolysis, and also to hazardous byproducts formed in the course of this chemical reaction. Therefore increasing concern over the  
20       use of flavoring agents obtained by chemical hydrolysis has lead to the development of enzymatic hydrolysis processes.

      Enzymatic hydrolysis processes aim at obtaining a high degree of hydrolysis (DH), and this is usually attained using a complex of unspecific acting proteolytic enzymes (i.e. unspecific acting endo- and exo-peptidases). In this way e.g. WO  
25       94/25580 describes a method for hydrolyzing proteins by use of an unspecific acting enzyme preparation obtained from *Aspergillus oryzae*. Specific acting proteolytic enzymes have not been used for this purpose, because such enzymes only lead to an inadequate degree of hydrolysis.

      Only relatively few specific acting proteolytic enzymes have been reported.  
30       Thus proteolytic enzymes preferentially cleaving peptides at glutamoyl-peptide bonds (Glu-specific proteases) obtained from *Staphylococcus aureus* [Drapeau G R, Boily Y and Houmard J; J. Biol. Chem. 1972 **247** (20) 6720-6726], from *Actinomyces* sp. [Mosolova O V, Rudenskaya G N, Stepanov V M, Khodova O M and Tsaplina I A; Biokhimiya 1987 **52** (3) 414-422], from *Streptomyces griseus* [Yoshida N, Tsuruyama S, Nagata K, Hirayama K, Noda K and Makisumi S; J. Biochem. 1988 **104** 451-456], from  
35       *Streptomyces thermovulgaris* [Khaldarova N Y, Rudenskaya G N, Revina L P,

Stephanov V M and Egorov N S; Biochimii Moscow Eng. 1989 **54** (1) 32-38], from *Bacillus subtilis* [Niidome T, Yoshida N, Ogata F, Ito A and Noda K; J. Biochem. 1990 **108** 965-970], and from *Bacillus licheniformis* [WO 91/13554 A1], have been reported.

Glu-specific proteases primarily find applications in studies of protein structures. However, use of a Glu-specific *Staphylococcus aureus* protease for modifying the solubility and structural properties of casein has been reported [Chobert J-M, Sitohy M Z and Whitaker J R; J. Agric. Food Chem. 1988 **36** 220-224], and WO 91/13554 A1 describes the use of the *Bacillus licheniformis* for obtaining a limited specific hydrolysis of proteins.

Whereas glutamine (Gln) is almost tasteless, glutamic acid (Glu), whether free or peptide bound, plays an important role for the flavor and palatability of protein hydrolysates. Glutamic acid may be formed by converting free glutamine to glutamic acid. This conversion (i.e. deamidation) is inherently taking place during conventional chemical hydrolysis using strong hydrochloric acid.

Alternatively glutamic acid may be formed from free glutamine by the action of a glutaminase (L-Glutaminase, EC 3.5.1.22 or D-Glutaminase, EC 3.5.1.35). However, substantial amounts of the glutamine may be lost as it spontaneously converts to pyroglutamine.

In contrast to glutaminase, peptidoglutaminase (PGases) acts on peptide bound glutamine. Two types of peptidoglutaminases are known. Peptidyl-glutaminase (EC 3.5.1.43; Peptidoglutaminase I) specific for glutamine substituted at the  $\alpha$ -amino group, and Protein-glutamine glutaminase (EC 3.5.1.44; Peptidoglutaminase II) specific for glutamine substituted at the carboxyl position or both the  $\alpha$ -amino and carboxyl positions. Peptidoglutaminases obtained from *Aspergillus japonicus*, *Bacillus circulans*, *Cryptococcus albidus*, and *Debaryomyces hansenii* have been reported [Kikuchi M & Sakaguchi K; Agr. Biol. Chem. 1973 **37** (4) 719-724].

Peptidoglutaminases are known and useful for modifying food proteins. Structural modifications of proteins improve their functional properties and extends their use as food ingredients. Thus US 5,082,672 describes a method of treating food proteins by deamidation with a peptidoglutaminase, by which method the solubility, emulsification, foaming and other functional properties of soy proteins become improved. However, US 5,082,672 also report that the ability of peptidoglutaminase to deamidate intact soy protein is limited due to its large molecular size and/or unique conformation.

Therefore US 5,082,672 teach initial degradation of the substrate by hydrolysis with Alcalase™, an un-specific acting endo/exo-peptidase complex obtained from *Bacillus*

*licheniformis*, and/or heat treatment, and find that pre- and post-heat treatment of hydrolyzed soy protein produced the greatest degree of deamidation.

US 3,857,967 describes a process for preparing food and beverages with a peptidoglutaminase obtained from *Bacillus circulans*. Also, in order to obtain the greatest  
5 degree of deamidation, US 3,857,967 teach initial degradation of the proteinaceous substrate by use of un-specific acting endo/exo-peptidases.

*Mimouni et al.* [*Mimouni B, Raymond J, Merle-Desnoyers AM, Azanza JL & Ducastaing A; Journal of Cereal Science* 1994 21 153-165] describe a combined acid deamidation and enzymatic hydrolysis for improvement of the functional properties of  
10 wheat gluten. More particularly, *Mimouni et al.* describe acid deamidation combined with the use of unspecific acting endo-peptidases.

### SUMMARY OF THE INVENTION

15 It has now been found that protein hydrolysates of excellent flavor and functionality can be obtained by subjecting the proteinaceous material to the combined action of a deamidation process and the action of a specific acting proteolytic enzyme.

Accordingly the present invention provides a method of obtaining from a proteinaceous substrate a hydrolysate enriched in free glutamic acid and/or peptide  
20 bound glutamic acid residues, which method comprises the steps of subjecting the substrate to a deamidation process; and subjecting the substrate to the action of a specific acting proteolytic enzyme.

In a preferred embodiment, the invention provides a method for obtaining from a proteinaceous substrate a hydrolysate enriched in glutamic acid, which method  
25 comprises the steps of subjecting the substrate to the action a peptidoglutaminase and subjecting the substrate to the action of a proteolytic enzyme preferentially cleaving glutamoyl-peptide bonds, followed by the action of one or more unspecifically acting endo- and/or exo-peptidase enzymes.

### 30 DETAILED DISCLOSURE OF THE INVENTION

The present invention relates to a method of obtaining a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, which method comprises the steps of

35 (i) subjecting the substrate to a deamidation process; and

(ii) subjecting the substrate to the action of a specific acting proteolytic enzyme.

The two steps, (i) and (ii), may be accomplished simultaneously, or step (ii) may be performed subsequent to step (i).

5 By the method of the invention protein hydrolysates of excellent flavor and functionality can be obtained. In particular protein hydrolysates of excellent flavor can be obtained because glutamic acid (Glu), whether free or peptide bound, plays an important role for the flavor and palatability of protein hydrolysates. However, by the method of the invention protein hydrolysates of improved functionality can also be obtained, in  
10 particular with respect to improved solubility, improved emulsifying properties, increased degree of hydrolysis and improved foaming properties.

### Deamidation Processes

The conversion of amides (glutamine or asparagine) into charged acids  
15 (glutamic acid or aspartic acid) via the liberation of ammonia is known as deamidation. Deamidation may take place as a non-enzymatic or as an enzymatic deamidation process.

In a preferred embodiment the deamidation is carried out as an enzymatic deamidation process. In particular the enzymatic may be carried out by subjecting the  
20 substrate to the action of a transglutaminase or the action of a peptidoglutaminase.

### Transglutaminases

In a preferred embodiment the deamidation is carried out as an enzymatic deamidation process in which the substrate is subjected to the action of a  
25 transglutaminase. The transglutaminase may be of any convenient source including those derived from mammals, see e.g. JP 1050382 and JP5023182, including activated Factor XIII, see e.g. WO 93/15234; those derived from fish, see e.g. EP 555,649; and those derived from microorganisms, see e.g. EP 379,606, WO 96/06931 and WO 96/22366.

30 In another specific embodiment, the transglutaminase is derived from an Oomycete, including a strain of *Phytophthora*, in particular *Phytophthora cactorum*, a strain of *Pythium*, in particular *Pythium irregulare*, *Pythium sp.*, *Pythium intermedium*, *Pythium ultimum*, and *Pythium perillum* (or *P. periplocum*).

In yet another specific embodiment, the transglutaminase is of bacterial origin  
35 and is preferably derived from a strain of *Bacillus*, in particular *Bacillus subtilis*, a strain of *Streptoverticillium*, in particular *Streptoverticillium mobaraensis*, *Streptoverticillium*

*griseocarneum*, *Streptoverticillium cinnamoneum*, and a strain of *Streptomyces*, in particular *Streptomyces lydicus*.

The transglutaminase shall be added to the proteinaceous substrate in effective amounts. The transglutaminase may be added in amounts conventionally  
5 employed in deamidation processes. It is at present contemplated that an effective amount of transglutaminase is in the range of from about 0.01 to about 5 % (w/w) of enzyme preparation relating to the amount of substrate, preferably in the range of from about 0.1 to about 1 % (w/w) of enzyme preparation relating to the amount of substrate.

The enzymatic treatment (incubation) may take place at any convenient  
10 temperature at which the enzyme preparation does not become inactivated, preferably in the range of from about 20°C to about 70°C.

In accordance with established practice the enzyme preparation may suitably be inactivated by increasing the temperature of the incubation mixture to a temperature where the enzymes become inactivated, e.g. to above about 70°C, or similarly by de-  
15 creasing the pH of the incubation mixture to a value where the enzymes become inactivated, e.g. below about 4.0.

### Peptidoglutaminases

In another preferred embodiment the deamidation is carried out as an  
20 enzymatic deamidation process in which the substrate is subjected to the action of a peptidoglutaminase.

In a more specific embodiment, the peptidoglutaminase used in the method of the invention may be a peptidoglutaminase I (peptidyl-glutaminase; EC 3.5.1.43), or a peptidoglutaminase II (protein-glutamine glutaminase; EC 3.5.1.44), or any mixture  
25 hereof. The peptidoglutaminase may be derived from a strain of *Aspergillus*, in particular a strain of *Aspergillus japonicus*, a strain of *Bacillus*, in particular a strain of *Bacillus circulans*, a strain of *Cryptococcus*, in particular a strain of *Cryptococcus albidus*, or a strain of *Debaryomyces*, in particular a strain of *Debaryomyces hansenii*.

The peptidoglutaminase shall be added to the proteinaceous substrate in  
30 effective amounts. The peptidoglutaminase may be added in amounts conventionally employed in deamidation processes. It is at present contemplated that an effective amount of peptidoglutaminase is in the range of from about 0.01 to about 100.000 PGase Units per 100 g of substrate, in particular in the range of from about 0.1 to about 10.000 PGase Units per 100 g of substrate.

The enzymatic treatment (incubation) may take place at any convenient temperature at which the enzyme preparation does not become inactivated, preferably in the range of from about 20°C to about 70°C.

In accordance with established practice the enzyme preparation may suitably  
5 be inactivated by increasing the temperature of the incubation mixture to a temperature where the enzymes become inactivated, e.g. to above about 70°C, or similarly by decreasing the pH of the incubation mixture to a value where the enzymes become inactivated, e.g. below about 4.0.

## 10 Specific Acting Proteolytic Enzymes

The method of the invention comprises the step of subjecting the substrate to the action of a specific acting proteolytic enzyme. More specifically the specific acting proteolytic enzyme may be a specific acting endo-peptidase or a specific acting exo-peptidase. The specific acting proteolytic enzyme may be of any available source.

15

### Endo-peptidases

In a preferred embodiment the specific acting proteolytic enzyme is an endo-peptidase such as

- (i) a glutamyl endopeptidase (EC 3.4.21.19);
- 20 (ii) a lysyl endopeptidase (EC 3.4.21.50);
- (iii) a leucyl endopeptidase (EC 3.4.21.57);
- (iv) a glycyl endopeptidase (EC 3.4.22.25);
- (v) a prolyl endopeptidase (EC 3.4.21.26);
- (vi) trypsin (EC 3.4.21.4) or a trypsin-like (lysine/arginine specific)  
25 endopeptidase; or
- (vii) a peptidyl-Asp metalloendopeptidase (EC 3.4.24.33).

The glutamyl endopeptidase (EC 3.4.21.19) of item (i) i.e. a proteolytic enzyme preferentially cleaving glutamoyl-peptide bonds, may preferably be derived from a strain of *Bacillus*, in particular *Bacillus licheniformis* and *Bacillus subtilis*, a strain of  
30 *Staphylococcus*, in particular *Staphylococcus aureus*, a strain of *Streptomyces*, in particular *Streptomyces thermovulgaris* and *Streptomyces griseus*, or a strain of *Actinomyces sp.*

The lysyl endopeptidase (EC 3.4.21.50) of item (ii) may preferably be derived from a strain of *Achromobacter*, in particular *Achromobacter lyticus*, a strain of  
35 *Lysobacter*, in particular *Lysobacter enzymogenes*, or a strain of *Pseudomonas*, in particular *Pseudomonas aeruginosa*.



The leucyl endopeptidase (EC 3.4.21.57) of item (iii) may be of plant origin.

The glycyl endopeptidase (EC 3.4.22.25) of item (iv) may preferably be derived from the papaya plant (*Carica papaya*).

The prolyl endopeptidase (EC 3.4.21.26) of item (v) may preferably be  
5 derived from a strain of *Flavobacterium*, or it may be of plant origin.

The trypsin-like endopeptidase of item (vi) may preferably be derived from a strain of *Fusarium*, in particular *Fusarium oxysporum*, e.g. as described in WO 89/06270 or WO 94/25583.

The peptidyl-Asp metalloendopeptidase (EC 3.4.24.33) of item (vii) may  
10 preferably be derived from a strain of *Pseudomonas*, in particular *Pseudomonas fragi*.

### Exo-peptidases

In another preferred embodiment the specific acting proteolytic enzyme is an exo-peptidase enzyme that may act from either ends of the peptide, i.e. may be a  
15 aminopeptidase or it may be a carboxypeptidase.

In a preferred embodiment, the specific acting proteolytic enzyme is an aminopeptidase such as

- (i) a leucyl aminopeptidase (EC 3.4.11.1); or
- (ii) a tripeptide aminopeptidase (EC 3.4.11.4).

20 In another preferred embodiment, the specific acting proteolytic enzyme is a carboxypeptidase such as

- (i) a proline carboxypeptidase (EC 3.4.16.2);
- (ii) a carboxypeptidase A (EC 3.4.17.1);
- (iii) a carboxypeptidase B (EC 3.4.17.2);
- 25 (iv) a carboxypeptidase C (EC 3.4.16.5);
- (v) a carboxypeptidase D (EC 3.4.16.6);
- (vi) a lysine(arginine) carboxypeptidase (EC 3.4.17.3);
- (vii) a glycine carboxypeptidase (EC 3.4.17.4);
- (viii) an alanine carboxypeptidase (EC 3.4.17.6);
- 30 (ix) a glutamate carboxypeptidase (EC 3.4.17.11);
- (x) a peptidyl-dipeptidase A (EC 3.4.15.1); or
- (xi) a peptidyl-dipeptidase (EC 3.4.15.5).

The specific acting endo- or exo-peptidase enzyme shall be added to the  
35 proteinaceous substrate in an effective amount. The proteolytic may be added in amounts conventionally employed in protein hydrolysis processes. It is at present contemplated that an effective amount of endo-peptidase is in the range of from about 0.05 to about

15 CPU/100 g of substrate, in particular in the range of from about 0.1 to about 5 CPU/100 g of substrate, and an effective amount of exo-peptidase is in the range of from about 0.001 to about 0.5 AU/100 g of substrate, in particular in the range of from about 0.01 to about 0.1 AU/100 g of substrate.

- 5           The enzymatic treatment (incubation) may take place at any convenient temperature at which the enzyme preparation does not become inactivated, preferably in the range of from about 20°C to about 70°C.

          In accordance with established practice the enzyme preparation may suitably be inactivated by increasing the temperature of the incubation mixture to a temperature  
10 where the enzymes become inactivated, e.g. to above about 70°C, or similarly by decreasing the pH of the incubation mixture to a value where the enzymes become inactivated, e.g. below about 4.0.

#### Unspecific Acting Proteolytic Enzymes

- 15           In a more specific embodiment, the method of the invention comprises the additional step of

(iii) subjecting the substrate to the action of one or more unspecific acting endo- and/or exo-peptidase enzymes.

          In this step the substrate is subjected to a conventional hydrolysis step. This  
20 additional step may take place simultaneously with steps (i) and (ii), or it may be accomplished subsequent to steps (i) and (ii). In particular the substrate may be the reaction mixture of steps (i) and (ii).

          In a preferred embodiment the unspecific acting endo- and/or exo-peptidase enzyme is derived from a strain of *Aspergillus*, in particular a strain of *Aspergillus niger*,  
25 a strain of *Aspergillus oryzae*, or a strain of *Aspergillus soyae*, or a strain of *Bacillus*, in particular a strain of *Bacillus amyloliquefaciens*, a strain of *Bacillus lentus*, a strain of *Bacillus licheniformis* or a strain of *Bacillus subtilis*.

          The unspecific acting endo- and/or exo-peptidase enzyme is added to the substrate in amounts in the range of from about 0.05 to about 15 CPU/100 g of  
30 substrate, in particular in the range of from about 0.1 to about 5 CPU/100 g of substrate.

          The enzymatic treatment (incubation) may take place at any convenient temperature at which the enzyme preparation does not become inactivated, preferably in the range of from about 20°C to about 70°C.

          In accordance with established practice the enzyme preparation may suitably  
35 be inactivated by increasing the temperature of the incubation mixture to a temperature where the enzymes become inactivated, e.g. to above about 70°C, or similarly by de-

creasing the pH of the incubation mixture to a value where the enzymes become inactivated, e.g. below about 4.0.

### Proteinaceous Substrates

5           The proteinaceous substrate subjected to the method of the present invention may consist of intact proteins, or it may consist of pre-hydrolyzed proteins (peptides), or it may be a mixture hereof. Also, the proteinaceous substrate may be vegetable or of animal origin.

          Preferably the proteinaceous substrate is of vegetable origin, and may in particular be soy protein, grain protein, e.g. wheat gluten, corn gluten, barley, rye, oat, 10 rice, zein, cotton seed protein, rape seed protein, peanut, alfalfa protein, pea protein, fabaceous bean protein, sesame seed protein, or sunflower.

          A proteinaceous substrate of animal origin may in particular be whey protein, casein, meat proteins, fish protein, red blood cells, egg white, gelatin, or lactoalbumin.

15

### Industrial Applications

          The hydrolysates enriched in free glutamic acid and/or peptide bound glutamic acid residues obtained by the method of the invention may be used in various industrial applications, in particular where there is a need for the incorporation of 20 functional proteins.

          Therefore, in another aspect, the present invention provides a food product comprising a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues obtained by the method of the invention.

          In yet another aspect, the invention provides an animal feed additive 25 comprising a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues obtained by the method of the invention.

### Assay for Enzymatic Activity

#### Peptidoglutaminase Activity

30           The peptidoglutaminase activity may determined according to the procedure of *Cedrangoro et al.* [*Cedrangoro et al.*; Enzymologia 1965 29 143], and as described in US 3,857,967.

          According to this method, 0.5 ml of an enzyme sample, adjusted to pH 6.5 with 1N NaOH, is charged into a small vessel. Following the procedure of *Cedrangoro et al.*, 1 ml of a borate buffer solution, pH 10.8, is added to the vessel, the discharged 35 ammonia is absorbed by 5N sulphuric acid, and the mixture is allowed to form colour by

use of Nessler's reagent, and the light absorptivity of the formed colour is measured at 420 m $\mu$ .

One PGase unit is the amount of enzyme capable of producing 1  $\mu$ mol/minute of ammonia under these conditions.

5 Alternatively, the peptidoglutaminase activity may be determined according to the procedure described in Example 2, below.

#### Proteolytic Activity: Casein Protease Units (CPU)

The proteolytic activity may be determined using casein as substrate. One  
10 Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 mM of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5.

A folder AF 228/1 describing this analytical method in more detail is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by  
15 reference.

#### Proteolytic Activity: Anson Units (AU)

The proteolytic activity may alternatively be determined with denatured haemoglobin as substrate. In the Anson-Haemoglobin method for the determination of  
20 proteolytic activity denatured haemoglobin is digested, and the undigested haemoglobin is precipitated with trichloroacetic acid (TCA). The amount of TCA soluble product is determined with phenol reagent, which gives a blue colour with tyrosine and tryptophan.

One Anson Unit (AU) is defined as the amount of enzyme which under standard conditions (i.e. 25°C, pH 7.5 and 10 min. reaction time) digests haemoglobin at  
25 an initial rate such that there is liberated per minute an amount of TCA soluble product which gives the same colour with phenol reagent as one milli-equivalent of tyrosine.

A folder AF 4/5 describing the analytical method in more detail is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by  
reference.

30

#### Proteolytic Activity: Leucine Amino Peptidase Units (LAPU)

The proteolytic activity may alternatively be determined with leucine-p-nitroanilide as substrate. Upon hydrolysis p-nitroanilide is liberated turning the solution yellow. The activity is determined using p-nitroanilide as chromophore, the yellow  
35 colour produced being photometered at 405 nm.

1 Leucine Amino Peptidase Unit (LAPU) is the amount of enzyme which decomposes 1  $\mu$ (micro)M substrate per minute at the following conditions: 26 mM of L-

leucine-p-nitroanilide as substrate, 0.1 M Tris buffer (pH 8.0), 40°C, 10 minutes reaction time.

## EXAMPLES

5

The invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

### Example 1

#### 10 Increased Protein Solubility and Release of Glutamate by Deamidation

##### Hydrolysis

Wheat gluten (WG) was obtained from Cargill (JOB 5141 ) and deamidated wheat gluten (DWG) was obtained from StaPro Consultancy B.V., Lemdijk 32, 9422  
15 TH Smilde, NL. Suspensions of 8 % protein were made by mixing 11 g of gluten with 89 g of water. The pH was adjusted to 6.5 with NaOH. Glutamate/aspartate specific protease (SP446), obtainable as described in WO 91/13554, or lysine/arginine specific protease (SP387) obtainable as described in WO 89/06270, was added to the suspensions. The dosage was 0.01 AU/g protein for SP446 and 0.006 AU/g protein for  
20 SP387. Flavourzyme™ (an un-specifically acting protease preparation available from Novo Nordisk A/S, Denmark, containing endo- and exo-peptidase activities, and obtained by fermentation of *Aspergillus oryzae*) was added to some of the hydrolysates at a dosage of 20 LAPU/g protein. The hydrolyses were carried out at 50°C without further pH adjustment for 18 hours. The enzymes were inactivated by  
25 heating at 85°C for 15 minutes. The pH was adjusted to 5 and the hydrolysates centrifuged. The content of protein and free glutamate in the supernatant was determined.

##### Determination of protein

30 Protein was determined by Kjeldahl analysis, using a Kjeldahl factor of 6.25.

##### Determination of glutamate

The content of free glutamate was determined by use of the Boehringer-Mannheim kit for glutamate determination (Cat. No. 139 092). The method was  
35 adapted for use in microtiter plates.

Results

Hydrolysate	Protein Solubility %		Glutamate Content mg/l	
	WG	DWG	WG	DWG
SP446	18	54	0	0
SP387	35	44	0	0
SP446+ Flavourzyme™	34	87	1000	2000

- 5 When comparing wheat gluten (WG) to deamidated wheat gluten (DWG), the results show that deamidation increase the susceptibility of the gluten to specific proteases, such that more protein becomes soluble. By addition of Flavourzyme™ on top of a specific protease the release of glutamate is doubled due to deamidation.

10 **Example 2****Enzymatic Deamidation and Release of Glutamate**Fermentation of *Bacillus circulans*

- 15 *Bacillus circulans* cultures of the strain ATCC 21590 were grown in shake flasks of 400 ml capacity containing 200 ml of a medium composed of 1% polypeptone; 0.5 % lactose; 0.025 %  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ ; 0.005 %  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ ; 0.025 %  $\text{KH}_2\text{PO}_4$ ; and 17%  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ , with the pH adjusted to 7.2. Fermentation took place at 30°C for 20 hours using a stirring of 270 rpm. The cells were harvested by centrifugation at 4000 rpm in 1 litre flasks. The cells were frozen.

20

Purification of Peptidoglutaminase II from *Bacillus circulans*

All steps were performed at room temperature.

- 25 The frozen *Bacillus circulans* cells were thawed and suspended in Lysis buffer (50 mM Tris/HCl; 25% (w/v) sucrose; 1 mM EDTA, pH 8.0) until a homogeneous suspension was obtained - 100 g wet cells per litre Lysis buffer. Lysozyme™ (Fluka 62971, 10 mg/ml) and DNase I (Sigma DN-25, 10 mg/ml) were dissolved in Lysis

buffer. 100 ml Lysozyme™ solution, 10 ml 1.0M MgCl<sub>2</sub>, and 1 ml DNase I solution were added per litre cell suspension. The enzymes were allowed to act for 1 hour.

The suspension was filtered through a Seitz depth filter plate and the filtrate was transferred to a 10 mM KH<sub>2</sub>PO<sub>4</sub>/NaOH, pH 8.0 (Buffer A) on a Sephadex G25 column (Pharmacia). The enzyme solution was applied to a SOURCE Q column (Pharmacia) equilibrated in Buffer A and eluted with a linear NaCl gradient (0 → 500mM) in Buffer A. Fractions from the column were analysed for Peptidoglutaminase II activity as described below and fractions with activity were pooled. The absorbance of the pooled fractions at 280 nm was 1.78, thus the protein content was estimated to 1.8 mg/ml.

The purity of the protein in the Peptidoglutaminase II pool was approx. 25 % as judged from a SDS-PAGE gel. Thus the preparation contained approximately 0.5 mg/ml of pure peptidoglutaminase II.

#### 15 Peptidoglutaminase Assay Method

The enzymatic activity is determined by measuring the ammonia formed during hydrolysis of  $\gamma$ -carboxyamide of N-tert-Butoxycarbonyl (N-t-BOC-Gln-Pro; SIGMA No. B-4403) using the Boehringer-Mannheim kit for ammonia determination (Cat. No. 1112732). In this kit, ammonia is measured by determination of the consumption of NADH by glutamate dehydrogenase, and blanks without the addition of N-t-BOC-Gln-Pro were also applied in order to subtract the effect of other NADH consuming enzymes.

#### Wheat Gluten Hydrolysis

25 200 mg of wheat gluten protein was added 9 ml of boiling water and after cooling, pH was adjusted to 7.0. Then 250  $\mu$ l of the peptidoglutaminase II preparation (PEP) described above was added. The glutamate/aspartate specific protease (SP446) from example 1 was added in an amount of 0.04 AU/g protein, and Flavourzyme™ from example 1 was added at in an amount of 20 LAPU/g protein.

30 Hydrolysis proceeded without pH adjustment for 18 hours at 50°C. Blanks without addition of peptidoglutaminase were produced. The hydrolysates were centrifuged and glutamate was measured as described in Example 1.

The degree of hydrolysis (DH) of the wheat gluten protein was determined as described below

Determination of the Degree of Hydrolysis (DH)

The DH of the protein, defined as described in by *Adler-Nissen* [*Adler-Nissen J; Enzymic Hydrolysis of Food proteins*; Elsevier Applied Science Publishers, 1986] was determined by reaction of the supernatant with OPA (ortho-phthalaldehyde, Sigma). For the OPA reagent, 160mg of OPA was dissolved in 4ml ethanol and transferred to a 200 ml volumetric flask containing a solution of 7.62 g di-sodium tetraborate decahydrate and 200 mg Sodium dodecylsulphate and 176 mg di-thiothreitol and the flask was filled to the mark with water. The reagent is light sensitive.

25 µl of suitably diluted supernatant was mixed with 200 µl OPA reagent in a microtiter plate well and allowed to react for exactly 2 minutes at 25°C. The absorbance at 340 nm was measured in a microtiter plate reader and compared to the absorbance of a 95 mM L-serine standard solution after subtraction of the blank value (water reacted with OPA-reagent). To find the true DH, the serine equivalents measured in the supernatants were corrected with the factors suggested by *Adler-Nissen* for the Trinitrobenzenesulfonic acid method [*Adler-Nissen J; Agricultural and Food Chemistry*, 1979 27 (6) 1256] which give the same response as the described OPA method. The degree of hydrolysis was calculated on basis of the total amount of protein in the hydrolysis mixture (not on basis of soluble protein).

Results

Hydrolysis	DH %	Glutamate mg/l
Minus PEP	40	131
Plus PEP	43	171

As it is seen from the table, hydrolysis with the peptidoglutaminase preparation increase the DH as well as the release of glutamate.



## CLAIMS

1. A method of obtaining from a proteinaceous substrate a hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, which method  
5 comprises the steps of
  - (i) subjecting the substrate to a deamidation process; and
  - (ii) subjecting the substrate to the action of a specific acting proteolytic enzyme.
- 10 2. The method according to claim 1, in which the deamidation process is carried out as a non-enzymatic deamidation.
3. The method according to claim 1, in which the deamidation process is carried out as an enzymatic deamidation.
- 15 4. The method according to claim 3, in which the enzymatic deamidation process is carried out by subjecting the substrate to the action of a transglutaminase.
5. The method according to claim 5, in which the transglutaminase is derived from an Oomycete, including a strain of *Phytophthora*, in particular *Phytophthora cactorum*,  
20 a strain of *Pythium*, in particular *Pythium irregulare*, *Pythium sp.*, *Pythium intermedium*, *Pythium ultimum*, and *Pythium peritum* (or *Pythium periplocum*).
6. The method according to claim 5, in which the transglutaminase is of bacterial  
25 origin and is preferably derived from a strain of *Bacillus*, in particular *Bacillus subtilis*, a strain of *Streptovorticillium*, in particular *Streptovorticillium mobaraensis*, *Streptovorticillium griseocaneum*, and *Streptovorticillium cinnamomeum*, and a strain of *Streptomyces*, in particular *Streptomyces lydicus*.
- 30 7. The method according to claim 3, in which the enzymatic deamidation process is carried out by subjecting the substrate to the action of a peptidoglutaminase.
8. The method according to claim 7, the peptidoglutaminase is a peptidoglutaminase I (peptidyl-glutaminase; EC 3.5.1.43), or a peptidoglutaminase II (protein-glutamine  
35 glutaminase; EC 3.5.1.44), or a mixture hereof.

9. The method according to either of claims 7-8, in which the peptidoglutaminase is derived from a strain of *Aspergillus*, in particular a strain of *Aspergillus japonicus*, a strain of *Bacillus*, in particular a strain of *Bacillus circulans*, a strain of *Cryptococcus*, in particular a strain of *Cryptococcus albidus*, or a strain of *Debaryomyces*, in particular a strain of *Debaryomyces hansenii*.
10. The method according to any of claims 7-9, in which the peptidoglutaminase is added to the substrate in amounts in the range of from about 0.01 to about 100.000 PGase Units per 100 g of substrate, in particular in the range of from about 0.1 to about 10.000 PGase Units per 100 g of substrate.
11. The method according to any of claims 1-10, in which the specific acting proteolytic enzyme is an endo-peptidase such as
- (i) a glutamyl endopeptidase (EC 3.4.21.19);
  - (ii) a lysyl endopeptidase (EC 3.4.21.50);
  - (iii) a leucyl endopeptidase (EC 3.4.21.57);
  - (iv) a glycyl endopeptidase (EC 3.4.22.25);
  - (v) a prolyl endopeptidase (EC 3.4.21.26);
  - (vi) trypsin (EC 3.4.21.4) or a trypsin-like endopeptidase; or
  - (vii) a peptidyl-Asp metalloendopeptidase (EC 3.4.24.33).
12. The method according to claim 11, in which the glutamyl endopeptidase of (i) is derived from a strain of *Bacillus*, in particular *Bacillus licheniformis* and *Bacillus subtilis*, a strain of *Staphylococcus*, in particular *Staphylococcus aureus*, a strain of *Streptomyces*, in particular *Streptomyces thermovulgaris* and *Streptomyces griseus*, or a strain of *Actinomyces* sp.
13. The method according to claim 11, in which the lysyl endopeptidase of (ii) is derived from a strain of *Achromobacter*, in particular *Achromobacter lyticus*, a strain of *Lysobacter*, in particular *Lysobacter enzymogenes*, or a strain of *Pseudomonas*, in particular *Pseudomonas aeruginosa*.
14. The method according to claim 11, in which the glycyl endopeptidase of (iv) is derived from the papaya plant (*Carica papaya*).

15. The method according to claim 11, in which the prolyl endopeptidase of (v) is derived from a strain of *Flavobacterium*.
- 5 16. The method according to claim 11, in which the trypsin-like endopeptidase of (vi) is derived from a strain of *Fusarium*, in particular *Fusarium oxysporum*.
17. The method according to claim 11, in which the peptidyl-Asp metalloendopeptidase (EC 3.4.24.33) of (vii) is derived from a strain of *Pseudomonas*, in particular  
10 *Pseudomonas fragi*.
18. The method according to any of claims 1-16, in which the specific acting proteolytic enzyme is an exo-peptidase enzyme.
- 15 19. The method according to claim 17, in which the exo-peptidase is an aminopeptidase such as
- (i) a leucyl aminopeptidase (EC 3.4.11.1); or
  - (ii) a tripeptide aminopeptidase (EC 3.4.11.4).
- 20 20. The method according to claim 17, in which the exo-peptidase is a carboxypeptidase such as
- (i) a proline carboxypeptidase (EC 3.4.16.2);
  - (ii) a carboxypeptidase A (EC 3.4.17.1);
  - (iii) a carboxypeptidase B (EC 3.4.17.2);
  - 25 (iv) a carboxypeptidase C (EC 3.4.16.5);
  - (v) a carboxypeptidase D (EC 3.4.16.6);
  - (vi) a lysine(arginine) carboxypeptidase (EC 3.4.17.3);
  - (vii) a glycine carboxypeptidase (EC 3.4.17.4);
  - (viii) an alanine carboxypeptidase (EC 3.4.17.6);
  - 30 (ix) a glutamate carboxypeptidase (EC 3.4.17.11);
  - (x) a peptidyl-dipeptidase A (EC 3.4.15.1); or
  - (xi) a peptidyl-dipeptidase (EC 3.4.15.5).

21. The method according to any of claims 7-10, of obtaining from a proteinaceous substrate a hydrolysate enriched in glutamic acid, which method comprises the steps of
- (i) subjecting the substrate to the action a peptidoglutaminase; and
  - 5 (ii) subjecting the substrate to the action of a proteolytic enzyme preferentially cleaving glutamoyl-peptide bonds (i.e. a glutamyl endopeptidase; EC 3.4.21.19)
22. The method according to any of claims 1-21, in which the specific acting proteolytic enzyme is added to the substrate in amounts in the range of from about 0.05 to about 15 CPU/100 g of substrate, in particular in the range of from about 0.1 to about 5 CPU/100 g of substrate.
23. The method according to any of claims 1-22, in which step (i) and step (ii) of Claim 1 is accomplished simultaneously.
24. The method according to any of claims 1-22, in which step (ii) of Claim 1 is accomplished subsequent to step (i) of Claim 1.
25. The method according to any of claims 1-24, which method comprises the additional step of
- (iii) subjecting the substrate to the action of one or more unspecific acting endo- and/or exo-peptidase enzymes.
26. The method according to claim 25, in which steps (i), (ii) and (iii) are accomplished simultaneously.
27. The method according to claim 25, in which step (iii) is accomplished subsequent to steps (i) - (ii).
28. The method according to any of claims 25-27, in which the unspecific acting endo- and/or exo-peptidase enzyme of step (iii) is derived from a strain of *Aspergillus*, in particular a strain of *Aspergillus niger*, a strain of *Aspergillus oryzae*, or a strain of *Aspergillus soyae*, or a strain of *Bacillus*, in particular a strain of *Bacillus*

*amyloliquefaciens*, a strain of *Bacillus lentus*, a strain of *Bacillus licheniformis* or a strain of *Bacillus subtilis*.

29. The method according to any of claims 25-28, in which the unspecific acting endo-  
5 and/or exo-peptidase enzyme is added to the substrate in amounts in the range of  
from about 0.05 to about 15 CPU/100 g of substrate, in particular in the range of  
from about 0.1 to about 5 CPU/100 g of substrate, or in amounts in the range of  
from about 0.001 to about 0.5 AU/100 g of substrate, in particular in the range of  
from about 0.01 to about 0.1 AU/100 g of substrate.
- 10 30. The method according to any of claims 1-29, in which the proteinaceous substrate  
is of vegetable origin, in particular soy protein, grain protein, e.g. wheat gluten, corn  
gluten, barley, rye, oat, rice, zein, cotton seed protein, rape seed protein, peanut,  
alfalfa protein, pea protein, fabaceous bean protein, sesame seed protein, or  
15 sunflower.
31. The method according to any of claims 1-29, in which the proteinaceous substrate  
is of animal origin, in particular whey protein, casein, meat proteins, fish protein,  
red blood cells, egg white, gelatin, or lactoalbumin.
- 20 32. A food product comprising a hydrolysate enriched in free glutamic acid and/or  
peptide bound glutamic acid residues obtained by the method according to any of  
claims 1-31.
- 25 33. An animal feed additive comprising a hydrolysate enriched in free glutamic acid  
and/or peptide bound glutamic acid residues obtained by the method according to  
any of claims 1-31.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00230

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC6: A23J 3/34, A23J 3/30 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: A23J		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, CAPLUS, DIALINDEX(FOODSCI)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0480104 A1 (PROTEIN TECHNOLOGIES INTERNATIONAL, INC.), 15 April 1992 (15.04.92), abstract, page 2, line 35 - line 43, page 3, line 50 - line 54, table 1, claims --	1-33
A	US 5082672 A (JAMEL S. HAMADA ET AL), 21 January 1992 (21.01.92) --	1-33
A	WO 9113554 A1 (NOVO NORDISK A/S), 19 Sept 1991 (19.09.91) --	1-33
A	US 3857967 A (MAMORU KIKUCHI ET AL), 31 December 1974 (31.12.74) --	1-33
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" criterion document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
28 August 1997		29 -08- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer  Sofia Nikolopoulou Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 97/00230

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>STN International, File CAPLUS, CAPLUS accession no. 1995:215075, Mimouni, B. et al: "Combined acid deamidation and enzymic hydrolysis for improvement of the functional properties of wheat gluten"; &amp; J. Cereal Sci. (1994), 20(2), 153-65</p> <p>-- -----</p>	1-33

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

06/08/97

International application No.

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